PROTEOMIC ANALYSIS OF PHOSPHORYLATED NUCLEAR PROTEINS UNDERSCORES NOVEL ROLES FOR RAPID ACTIONS OF RETINOIC ACID IN THE REGULATION OF mRNA SPLICING AND TRANSLATION

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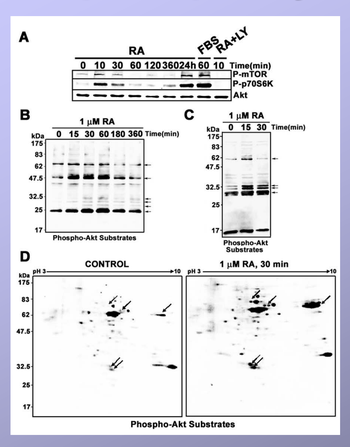
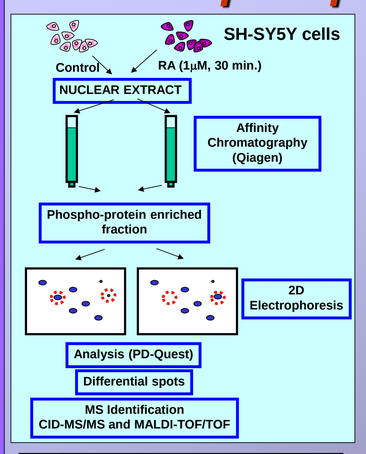
Retinoic Acid (RA) signaling is mediated by the Retinoic Acid Receptor (RAR), belonging to the Nuclear Hormone Receptor superfamily. In addition to its classical transcriptional actions, RAR also mediates rapid transcription-independent actions (also called non-genomic), consisting in the activation of signal transduction pathways, as the phosphatidyl-inositol-3-kinase (PI3K) or the ERK MAP-kinase signaling pathways. RA-induced rapid actions play roles in different physiological contexts. As an effort towards understanding the functions of those rapid actions on signaling elicited by RA, we have identified nuclear proteins whose phosphorylation state is rapidly modified by RA treatment in neuroblastoma cells, using a proteomic approach. Our results show that RA treatment led to changes in the phosphorylation patterns in two families of proteins: (i) those related to chromatin dynamics in relation to transcriptional activation, and (ii) those related to mRNA processing and in particular mRNA splicing. We show that treatment of neuroblastoma cells with RA leads to alteration of the regulation of pre-mRNA splicing and mRNA translation, via the activation of signaling pathways. Thus, our results underscore novel functions for the non-genomic signaling elicited by RAR in the regulation of mRNA processing, as part of a cellular response orchestrated by the nuclear receptor RAR. The results shown here contribute to the idea that transcriptional and transcription-independent actions elicited by nuclear hormone receptors are integrated and converge at multiple levels in the regulation of gene expression.

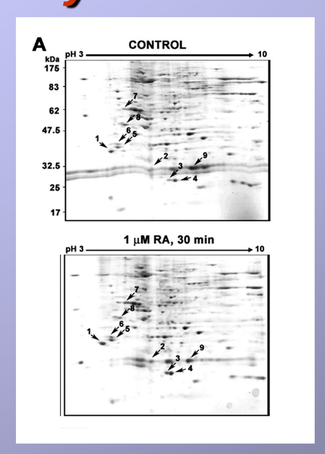
1. Short RA treatments lead to modifications in the phosphorylation patterns of nuclear proteins

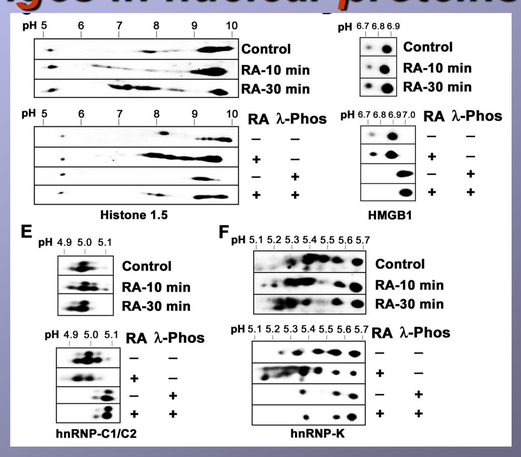


Short RA treatments induce the activation of the PI3K signalling pathway and its downstream components mTOR and p70S6 Kinase (Fig. 1A). Western blot with an antibody against phospho-serine/threonine residues within Akt kinase phosphorylation consensus site (RXRXXS/T), revealed that RA treatment increased rapidly the phosphorylation of several Akt substrate proteins, detectable in whole cell extracts (Fig. 1B), as well as in nuclear extract (Fig. 1C). 2-DE western blots demonstrate that RA treatment leads to an increase in the number and intensity of the spots corresponding to phosphorylated Akt substrates in nuclear extracts from neuroblastoma cells (Fig. 1D).

2. A proteomic approach to the identification of RA-induced phosphorylation changes in nuclear proteins



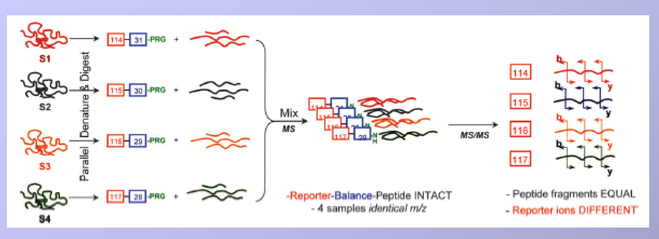




Spot no.	Name (symbol)
1	Nucleophosmin (NPM)
2, 9	Histone H1.5
3,4	High Mobility Group Box 1 (HMGB1)
5, 6	Heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNPC1/C2)
7	Heterogeneous nuclear ribonucleoprotein K (hnRNPK)
8	Polyadenylate binding Protein-2 (PABP2)

Nuclear phospho-proteins from cells treated for 30 min with RA or vehicle were prepared by affinity chromatography. The two samples were electrophoresed in 2D-gels and the patterns of spots compared. Differentially phosphorylated proteins were isolated and identified by Mass Spectrometry. RA-induced phosphorylation was validated in 2D-western blot.

3. Analysis of RA-induced differentially phosphorylated proteins trough the iTRAQ assay



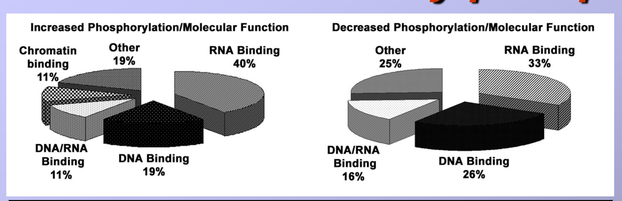
The iTRAQ method is based in the labeling of peptide fractions with reporter tags of different masses. We run in parallel the affinity-purified nuclear phospho-proteins from cells treated during 0, 15 and 30 min with RA. After denaturation and trypsin digestion, the samples were labeled and mixed The peptides were separated by multi-dimensional nano-liquid chromatography, and thedifferent fractions analyzed by tandem Mass Spec. The proteins are identified on the basis of peptide fragments, and a relative quantification is obtained from the relative levels of each reporter tag.

Phosphorylation increased by RA	
Chromobox protein homolog 1 (HP1 beta)	Arsenite-resistance protein 2 (ARS2)
Chromobox protein homolog 3 (HP1 gamma)	Barrier-to-autointegration factor (BAF)
Chromobox protein homolog 5 (HP1 alpha)	Complement component 1 Q
Heterogeneous nuclear ribonucleoprotein hnRNP A/B	Debrin
Heterogeneous nuclear ribonucleoprotein hnRNP A1	Nuclear protein Hcc-1
Heterogeneous nuclear ribonucleoprotein hnRNP A3	Nucleolin (C23)
Heterogeneous nuclear ribonucleoprotein hnRNP D0	Nucleophosmin
Heterogeneous nuclear ribonucleoprotein hnRNP G	HRP-3-Hepatoma-derived growth factor-related protein 3
Heterogeneous nuclear ribonucleoprotein hnRNP K	PC4 and SFRS1-interacting protein
Heterogeneous nuclear ribonucleoprotein hnRNP Q	Protein DEK
Heterogeneous nuclear ribonucleoprotein hnRNP A2 /B1 (*)	Protein RED
High mobility group protein B1 (HMGB1)	Protein SET
High mobility group protein B2 (HMGB2)	RNA-binding protein 8A
High mobility group protein HMG-I/HMG-Y (HMGA1)	Scaffold attachment factor B1 (SAFB1)
Splicing factor, arginine/serine-rich 1, SF2/ASF (SFRS1)	Splicing factor U2AF2
Splicing factor, arginine/serine-rich 2, SC35 (SFSR2)	Tropomyosin (1,2,3 or 4)
Splicing factor, arginine/serine-rich 3, SRp20 (SFRS3)	U1 small nuclear ribonucleoprotein 70 kDa (U1 snRNP 70 kDa)
Splicing factor, arginine/serine-rich 7, 9G8 (SFRS7)	U4/U6.U5 tri-snRNP-associated protein 1 (SART1)
Splicing factor, arginine/serine-rich 9, SRp30C (SFRS9)	

Phosphorylation reduced by RA	
ATP-dependent DNA helicase 2 subunit 1 (70 kDa subunit) (Ku70)	Histone H3 (1,2 or 3)
ATP-dependent DNA helicase 2 subunit 2 (80 kDa subunit) (Ku80)	Histone H4
ATP-dependent RNA helicase A (DHX9)	Histone-binding protein RBBP4
ATP-dependent RNA helicase DDX39	Lamin-B1
Probable ATP-dependent RNA helicase DDX5	Non-POU domain-containing octamer-binding protein
Spliceosome RNA helicase BAT1	Nucleolar protein 5 (NOP5)
Nucleolar RNA helicase 2	Nuclear mitotic apparatus protein 1 (NUMA1)
Heterogeneous nuclear ribonucleoprotein U (hnRNP U)	Splicing factor, proline- and glutamine-rich (SFPQ)
Heterogeneous nuclear ribonucleoprotein U-like protein 1 (hnRNP U-like 1)	Protein RRP5 homolog
Heterogeneous nuclear ribonucleoprotein hnRNP A2 /B1 (*)	RNA-binding protein Raly
60S ribosomal protein L22	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5 (SMARCA5)
Bromodomain adjacent to zinc finger domain protein 1B	Tubulin alpha chain (A,B or C)
General transcription factor II-I (GTFII-I, TFII-I)	U5 small nuclear ribonucleoprotein 200 kDa helicase (U5-200KD)

The iTRAQ assay resulted in the identification of 63 proteins whose phosphorylation changed as effect of RA treatment.

4. iTRAQ: GeneOntology analysis of RA-induced differentially phosphorylated proteins



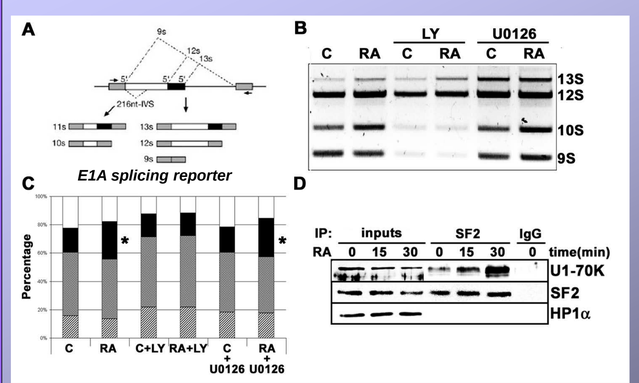
GO Categories	Increased Phosphorylation %	Decreased Phosphorylation %
RNA Metabolism (GO5)	68	63
RNA splicing (GO7)	41	31
Transcriptional Regulation (GO7)	27	38
RNA splicing, via trans-esterification reactions with bulged adenosine residues (GO9)	13	15

Among the proteins whose phosphorylation is modified by RA, the proteins involved in RNA metabolism are majority. The percentage of proteins involved in mRNA splicing is remarkable. Therefore we have postulated the following

HYPOTHESIS:

RA, trough the rapid activation of signaling pathways could regulate mRNA processing/splicing as part of a cellular response orchestrated by the nuclear receptor RAR

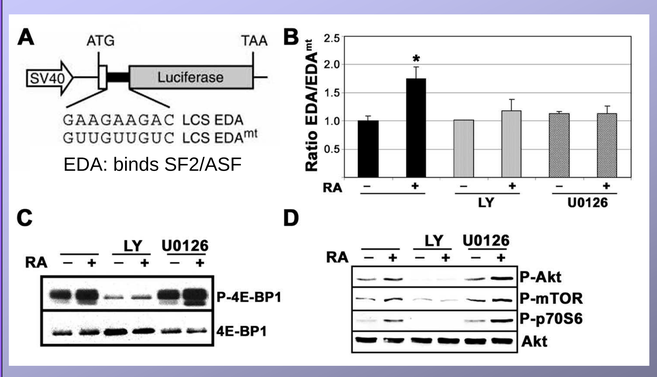
5. RA influences the regulation of alternative splicing via activation of signaling pathways



Using a splicing reporter minigene based on E1A (Fig. 5A) we could show that RA treatment alters the pattern of alternative splicing. This regulation is sensitive to PI3K inhibitors, but not to MAP kinase inhibitors. (Figs. 5B and C).

The interaction betweenthe SR protein SF2/ASF and the U1-70K spliceosome protein is increased by RA treatment, as shown in immunoprecipitation experiments (Fig. 5D)

6. RA regulates translation through the activation of signaling pathways

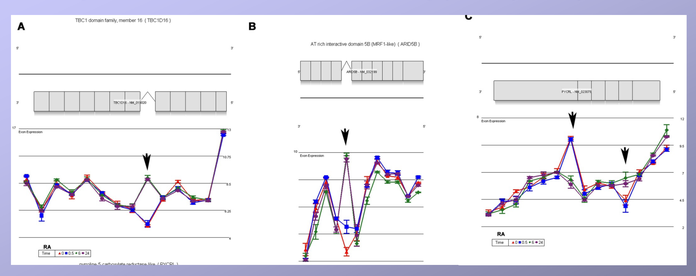


By using a translation reporter gene containing a binding site for SF2/ASF (Fig. 6A) we show RA-induced increase in translation, that was sensitive to inhibitors of both PI3K and ERK-MAP kinase pathways (Fig.6B).

RA increases the phosphorylation of 4E-BP1, an inhibitor of cap-binding eIF4E, through the activation of PI3K (Fig. 6C). However additional mechanisms must exist to explain the effects of MEK inhibitor U0126, that does not interferes the activation of PI3K pathway (Fig. 6D).

7. RA treatment modifies the alternative splicing pattern on endogenous genes in neuroblastoma cells

Affymetrix Human Gene 1.0 ST microarray (exon-specific probes)

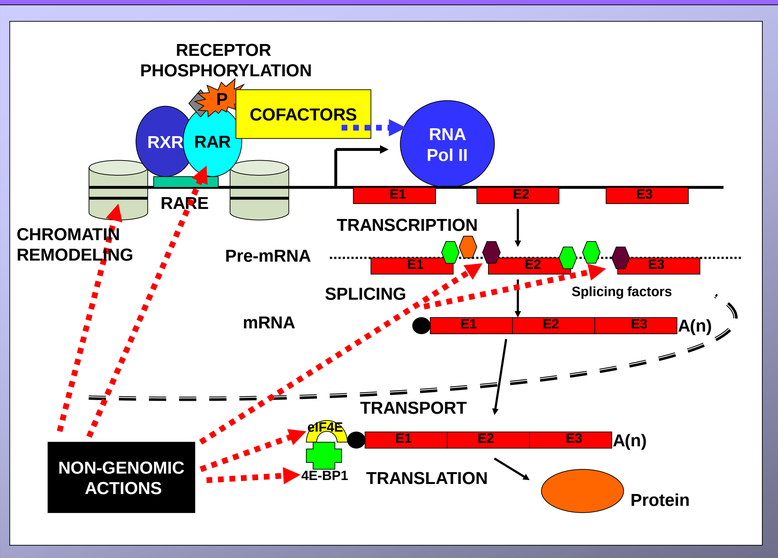


TBC1D16
RA-induced exon inclusion

ARID5B
RA-induced exon inclusion

PYCRL
RA-induced exon skipping
RA-induced exon inclusion

Preliminary data obtained with Affymetrix Human Gene 1.0ST microarray, which uses exon-specific probes, indicate that RA-induced changes in the alternative splicing pattern take place in a significant number of genes. The figure shows examples of RA-induced exon-inclusion as well as a complex pattern including both exon inclusion and skipping induced by RA



8. Conclusions

We provide the first evidence that RA, through the activation of signalling pathways, could regulate mRNA splicing and translation.

The results shown here contribute to the idea that transcriptional and transcription-independent actions elicited by nuclear hormone receptors are integrated and converge at multiple levels in the regulation of gene expression.

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